

Infrared nanospectroscopy study of the light-induced conformational changes of Channelrhodopsin

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Abstract. The channelrhodopsin-ChR2 is a light-sensitive transmembrane protein that acts as a selective ion channel between the intra- and the extra-cellular environments. In the last decade, ChR2 has proven to be essential for optogenetics, because, if expressed in mammalian neural cells, it enables the control of neuronal activity in response to visible light. Mid-infrared difference spectroscopy can probe the functional conformational changes of light-sensitive proteins, however intrinsic limitations of standard IR spectroscopy in terms of diffraction, and therefore number of probed proteins, require that the mid-IR experiments be performed on huge numbers of lipid membrane patches with overexpressed proteins. In this work, we apply for the first time IR difference nanospectroscopy, based on the use of mid-IR lasers and an atomic force microscope (AFM), to single membrane patches containing ChR2, obtaining relevant spectroscopy results for optogenetic applications and, more generally, for future experimental studies of light-sensitive proteins at the nanoscale.

1 Introduction

Light-sensitive microbial rhodopsins are proteins that play a crucial role in the cell functions acting as ion pumps or channels across the cell membrane in response to absorption of visible light. Although their native environment belongs to the world of archaea and bacteria, in the last decades the deep understanding of the molecular mechanisms that allow their functions has led to their application in diverse fields such as bioelectronics and neuroscience, depending on the specific protein and corresponding mutants.

The activity of light-sensitive proteins is triggered by photons in the visible range at a specific wavelength, which are absorbed by a retinal cofactor, covalently bounded to the protein structure, typically triggering a trans-to-cis isomerization. Starting from this event the protein undergoes a sequence of states conformationally and spectroscopically distinguishable that constitute the photocycle, during which the pump or the channel is active, until the initial state (i.e. the dark-adapted state) is recovered.

In this context, infrared (IR) spectroscopy in difference-absorption mode permits to investigate the functional conformational changes of the proteins relying on the vibrational excitation of the chemical bonds [1]. Conventional IR spectroscopy is limited in terms of sensitivity and it requires large amounts of proteins to be probed, preventing its application to the study of the single cell membrane patch.

We present an innovative IR difference spectroscopy approach at the nanoscale, based on the photothermal-induced resonance (PTIR) method, also called AFM-IR [2], to overcome the IR microscopy limitations in studying the light-sensitive conformational changes.

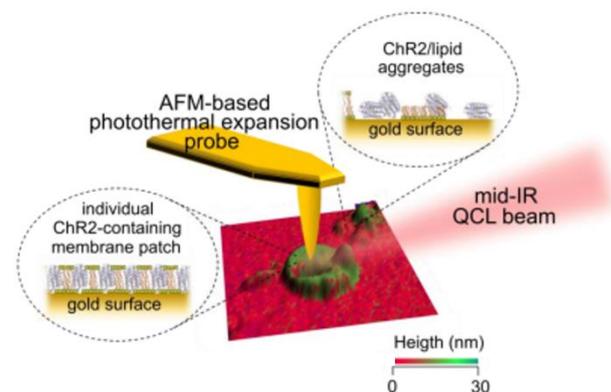


Fig. 1. Sketch of the AFM-IR setup based on a gold-coated tip probe and a mid-IR QCL laser beam. The AFM tip allows the topographic identification of a single membrane patch embedding ChR2 molecules, featuring a flat top surface and a height of ≈ 7 nm.

We are interested in the specific case of the light-activated conformational changes of the channelrhodopsin-ChR2 [3], an ion-gate protein used in the field of optogenetic to control neuronal stimuli with light in genetically modified neurons. Although

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optogenetics is now widely employed [4], a method of analysis of the detailed conformational changes of light-sensitive proteins at the single membrane level is obviously required to understand the opening/closing of the optogenetic gate in neurons. We then propose IR nanospectroscopy as an innovative method to study the protein activity at the level of a single cell membrane patch immobilized on a solid substrate. This configuration, in a broad sense, is more similar to that of neuronal cells with respect to the more widely studied sets of proteins in an aqueous suspension, and therefore it may provide meaningful information for optogenetic applications that sometimes require direct contact between the neuronal cells and metal probes.

2 Experimental

The sample investigated is prepared from a liquid suspension containing membrane patches loaded with ChR2 proteins. A microliter-size drop is cast on an ultra-flat gold surface and it is left to dry in a humidity-controlled atmosphere.

The AFM-IR vibrational imaging and nanospectroscopy are performed using a NanoIR2 (Anasys Instruments / Bruker Nano Surfaces) based on the photothermal expansion effect (see Fig.1 for a sketch of the experiment). The mid-IR laser source is an external-cavity broadly tunable quantum cascade laser (QCL) with a spectral range 900-1800 cm^{-1} (MIRcat by Daylight Solutions / Leonardo DRS). The AFM-IR technique exploits the photothermal expansion measured by the AFM readout circuit: when the QCL emits at a wavelength that matches one of the vibrational bands of the sample, the latter heats up and expands, exerting an extra force on the AFM tip that triggers the excitation of the natural cantilever bending modes. The amplitude of the cantilever oscillation is proportional to the absorption coefficient of the sample, and one can therefore obtain its IR vibrational spectrum by monitoring the deflection of the AFM probe [5]. The use of a gold-coated tip induces a strong plasmonic field enhancement at the apex, which allows one to collect IR response from a sample volume smaller than the diffraction limit by many orders of magnitude. The reason for that is the confinement of the temperature increase to the very small volume below the tip apex (thermoplasmonic effect). Furthermore, sensitivity down to the molecular monolayer can be reached by exploiting the plasmonic field enhancement in the nanogap between the gold-coated tip and a metallic surface used as support for the monolayer sample, in our case the single membrane patch [5,6]. In our experiment, AFM-IR difference nanospectroscopy is performed by implementing an external system of visible illumination to control protein photocycle based on two LEDs: a blue one (470 nm) to excite the ChR2 photocycle and a green one (505 nm) in order to recover the rest state of the proteins. The AFM-IR difference-spectra ΔA are obtained by averaging over 15 differences $A_{blue}(\omega) - A_{green}(\omega)$, where $A_{blue}(\omega)$ and $A_{green}(\omega)$ are the AFM-IR spectra acquired under blue and green light, respectively.

We perform micro-FTIR measurements on the same sample cast on an ultraflat gold surface to validate the difference-absorbance results obtained at the nanoscale and to highlight some differences between the nanospectroscopy experiment and the one realized probing a micrometer area of sample, i.e. a huge number of proteins (more than 10^7) embedded in stacked lipid membranes. The experiment has been realized at the synchrotron radiation source BESSY II of Helmholtz-Zentrum Berlin using an internal IR source of a Nicolet Continuum Infrared Microscope with a knife-edge aperture of $100\mu\text{m} \times 200\mu\text{m}$. The micro-FTIR difference-spectra ΔA were calculated as $\Delta A = A_{blue} - A_{green} = \log(R_{green}/R_{blue})$, where R_{blue} and R_{green} are the reflection spectra obtained under illumination at 470 nm and 505 nm, respectively.

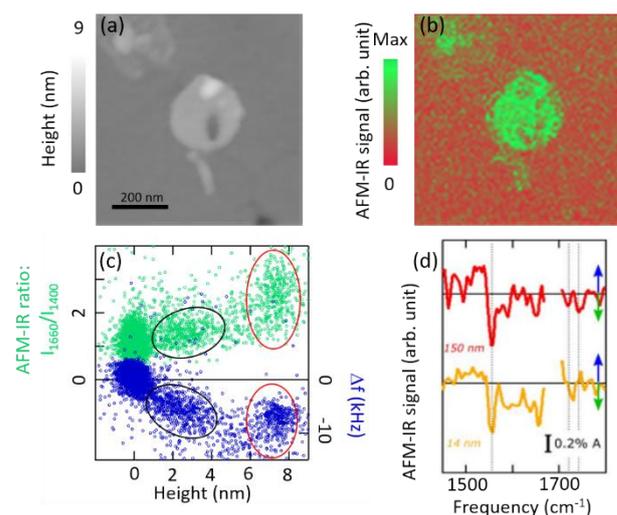


Fig. 2. (a) AFM topography of a single ChR2 membrane patch. (b) AFM-IR map acquired at 1660 cm^{-1} normalized by the map acquired at 1400 cm^{-1} . (c) Scatter plot of the ratio I_{1660}/I_{1400} (green dots) and of the variation of the resonance frequency $\Delta f = f - f_{Au}$ of the tip (blue dots) as a function of topographic height. (d) AFM-IR difference-absorbance spectra obtained on 150 nm-thick (red curve) and 14 nm-thick (yellow curve) areas of the ChR2 sample.

3 Results

The use of an AFM-based platform enables the identification of sample areas with membrane patches adhering to the ultraflat metal surface, in which the light-induced activity of proteins can then be properly investigated. In Fig. 2a we report the AFM topography map of a representative lipid membrane flake containing ChR2, whose thickness is less than 7 nm, confirming the adhesion of a single membrane patch to the substrate. IR nanoimaging may also provide information on the homogeneity of mechanical and spectroscopic properties of the assemblies. We indeed report an AFM-IR map acquired at 1660 cm^{-1} (in Fig. 2b) normalized by that acquired at a frequency that does not correspond to any IR absorption of the membrane (1400 cm^{-1}), so as to remove the background originating from thermal expansion of the

gold-coated tip and of the substrate. Moreover, in Fig.2c we show the scatter plot as a function of the thickness of the sample of (i) the ratio I_{1660}/I_{1400} of the AFM-IR map (green dots), where I is the photothermal expansion signal intensity, and of (ii) the stiffness variation (blue dots) in the investigated area. For the latter one we report in particular the variation of the resonance frequency $\Delta f = f - f_{Au}$ of the tip in contact with the sample respect to the frequency f_{Au} in contact with the bare ultraflat gold substrate. Each plot has one cluster centred at a null value of AFM height that represents the bare ultraflat gold surface and a second cluster centred at height value of 7 nm for the ChR2 samples (red ellipses in Fig.2c). There is also a third cluster (black ellipses in Fig.2c), whose pixels correspond to the top-left corner of Fig. 2b: the lower value of both the mean height and the absolute value of Δf of this cluster compared to the others, suggests the simultaneous presence on the gold surface of unoriented proteins and lipid aggregates (see also Fig. 1). The capability of discarding non-membrane lipid-protein aggregates is a characteristic of the AFM-IR nanoimaging method that cannot be matched by standard FTIR spectroscopy, even if employed in microscopy mode at a synchrotron.

After the identification of sample areas with single flat membrane patches on the ultraflat gold surface, we also use the AFM-IR platform to perform a functional study of ChR2 by probing its light-activated conformational changes connected to the ion channel function, measuring the difference-absorbance ($\Delta A = A_{blue} - A_{green}$) of the sample between the blue and the green illumination state. We perform nano-IR spectroscopy on both a 150 nm-thick area and a double membrane structure (14 nm-thick). As shown in Fig. 2d, the ΔA curves display common features known to be related to the vibrational modes involved in the protein activity, including the sharp C=C retinal stretching vibration peak (around 1555 cm^{-1}) and the two negative peaks in the carbonyl region around 1720 cm^{-1} and 1736 cm^{-1} . The comparison with the micro-FTIR spectroscopy measurements also confirms the validity of the nanoscale approach. Beyond these common features, there are slight differences between the two spectra in Fig. 2d that can be attributed to the direct adhesion of the membrane patch to the gold surfaces in the case of the 14-nm thick sample (yellow curve), which should have an effect on the ChR2 conformations during the photocycle [7].

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